SUMMARY

1. Coumarin when fed to rabbits is hydroxylated to 3-, 7- and 8-hydroxycoumarins, which are excreted in conjugation. This also occurs in the ferret, guinea pig, mouse and rat, the first three of which also excrete 5-hydroxycoumarin. However, not more than 25 % of the coumarin fed (dose 0.1 g./kg.) can be accounted for as conjugated glucuronic acid and ethereal sulphates excreted in the urine.

2. The ethereal sulphate and glucuronide of 3-hydroxycoumarin were isolated from the urine of rabbits fed on coumarin. 7-Hydroxycoumarin was also isolated after acid hydrolysis of the urine. 5- and 8-Hydroxycoumarins were not isolated but were detected in the hydrolysed urine by paper chromatography. The hydroxylation of coumarin has also been carried out with Fenton's reagent and an ascorbic acid-iron system. The results, which were not exactly the same as those found *in vivo*, have been discussed.

3. *o*-Coumaric acid appears to be excreted unchanged to a large extent by rabbits. It is, however, directly conjugated to a small extent since its ether glucuronide, *o*-glucosidurono-*trans*-cinnamic acid, was isolated and its glycine conjugate, *o*-hydroxy-*trans*-cinnamoylglycine, was detected by paper chromatography in the urine.

4. *o*-Coumaric acid also cyclized *in vivo*, since small amounts of the glucuronide of 4-hydroxycoumarin were isolated from the urine and 7-hydroxycoumarin was detected chromatographically. Melilotic acid, the corresponding saturated acid (dihydrocoumaric acid), also yields 4- and 7-hydroxycoumarin in the rabbit.

5. The mechanism of the cyclization of *o*-coumaric acid has been discussed and it is suggested that the coumarin derivatives formed

arise from β -oxidation products of *o*-coumaric acid.

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REFERENCES

- Boscott, R. J. & Bickel, H. (1954). *Biochem. J.* **56**, i.
 Boyland, E. & Sims, P. (1953). *J. chem. Soc.* p. 2966.
 Bray, H. G., Hybs, Z. & Thorpe, W. V. (1951). *Biochem. J.* **48**, 192.
 Burns, J. J., Weiner, M., Simson, G. & Brodie, B. B. (1953). *J. Pharmacol.* **108**, 33.
 Burns, J. J., Wexler, S. & Brodie, B. B. (1953). *J. Amer. chem. Soc.* **75**, 2345.
 Elderfield, R. C. (1951). In *Heterocyclic Compounds*, vol. 2, p. 214. New York: Wiley.
 Feigl, F., Feigl, H. E. & Goldstein, D. A. (1955). *J. Amer. chem. Soc.* **77**, 4162.
 Houben, J. & Pfankuch, E. (1926). *Ber. dtsch. chem. Ges.* **59**, 1598.
 Hough, L. (1954). In *Methods of Biochemical Analysis*, vol. 1, p. 231. London: Interscience Ltd.
 Hunter, R. B. & Shepherd, D. M. (1955). *Brit. med. Bull.* **11**, 56.
 Isaac, S. (1955). *C.R. Acad. Sci., Paris*, **240**, 2534.
 Kamil, I. A., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 235.
 Mead, J. A. R., Smith, J. N. & Williams, R. T. (1955). *Biochem. J.* **61**, 569.
 Mead, J. A. R., Smith, J. N. & Williams, R. T. (1958). *Biochem. J.* **68**, 63.
 Parke, D. V. (1956). *Biochem. J.* **62**, 339.
 Parke, D. V. & Williams, R. T. (1956). *Biochem. J.* **63**, 12P.
 Smith, J. N. & Williams, R. T. (1949). *Biochem. J.* **44**, 242.
 Smith, J. N. & Williams, R. T. (1950). *Biochem. J.* **46**, 243.
 Udenfriend, S., Clark, C. T., Axelrod, J. & Brodie, B. B. (1954). *J. biol. Chem.* **208**, 731.
 Vasiliu, H., Timosencu, A., Zaimov, C. & Coteleu, V. (1938). *Bul. Fac. Sti. agric. Chişinău*, **2**, 56.

The Preparation and Properties of Lithium Hydroxypyruvate and Hydroxypyruvic Acid

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Interest in the possible biological importance of hydroxypyruvate has been revived by the recent demonstrations that it acts as an effective substrate for the L-lactic dehydrogenases of muscle (Meister, 1952) and yeast (Dickens & Williamson, 1956*a*), and for the specific D-glyceric dehydrogenase (hydroxypyruvate reductase) present in certain higher plants (Stafford, Magaldi & Vennesland,

1954), being thus reduced respectively to L- or D-glyceric acid. Hydroxypyruvate is also a substrate for transketolase (Horecker, Smyrniotis & Klenow, 1953; Haba, Leder & Racker, 1955) and for carboxylases of yeast (Dickens & Williamson, 1956*b*) and of muscle (Dickens & Williamson, unpublished work). A transaminase present in animal tissues is able to transfer the amino group of L-alanine to

hydroxypyruvate, yielding L-serine and pyruvate (Sallach, 1955, 1956). 3-Phosphohydroxypyruvate has also recently been synthesized (Ballou & Hesse, 1956) and its biological behaviour studied (Ichihara & Greenberg, 1957). Hitherto, pure and adequately characterized preparations of hydroxypyruvic acid or of its salts have not been available, and biological studies on hydroxypyruvate have been made directly on the solution obtained (Sprinson & Chargaff, 1946) by the action of two equivalents of sodium hydroxide on bromopyruvic acid (I). Such solutions contain, we find, besides the sodium bromide, sufficient unhydrolysed bromopyruvate to inhibit sensitive sulphhydryl enzyme systems. Nevertheless, this work of Sprinson & Chargaff (which clearly indicates some of the serious uncertainties surrounding earlier preparations described as hydroxypyruvate) has been of great value in the subsequent biological studies.

The advantages of a pure crystalline salt of hydroxypyruvate, both for enzymic experiments and as an analytical standard, have now been achieved by the preparation of the lithium salt [Fig. 1 (II)], which proves to be the monohydrate,

$\text{CH}_2(\text{OH})\cdot\text{C}(\text{OH})_2\cdot\text{CO}_2\text{Li}$. Evidence is provided in the Addendum by Bellamy & Williams that the above formula correctly represents the constitution of this salt, rather than $\text{CH}_2(\text{OH})\cdot\text{CO}\cdot\text{CO}_2\text{Li} + \text{H}_2\text{O}$, since no free carbonyl group, or molecule of water of crystallization, is detected by infrared spectroscopy. It would therefore correspond in this respect with compounds such as chloral hydrate, $\text{CH}(\text{OH})_2\cdot\text{CCl}_3$, as is discussed in the Addendum. These authors also find that a similar structure applies to the monohydrated lithium salt of pyruvic acid, $\text{CH}_3\cdot\text{C}(\text{OH})_2\cdot\text{CO}_2\text{Li}$. In aqueous solutions containing ionized carboxyl groups these hydrated forms (diols) derived from the keto groups of the free acids will also be expected to be present.

Free hydroxypyruvic acid has not hitherto been obtained as an unequivocal preparation of the pure acid. The best crude preparations are probably those described by Sprinson & Chargaff (1946), by ether extraction made after acidification of concentrated aqueous solutions of the neutralized bromopyruvic acid. These oily products contained (by titration) 80–90 % of hydroxypyruvic acid. Fenton & Wilks (1912) prepared a 'basic lead salt' from the

Fig. 1. All melting points are with decomposition.

crude oxidation mixture resulting from the action of hydrogen peroxide and ferrous salt upon glyceric acid. The composition assumed for this salt (and for the calcium salt also prepared by them) is based solely on the metal content of the hydrated salt, and is probably therefore unreliable. (The evidence based on their preparation of a semicarbazone compound is discussed below.) From this lead salt an acid was prepared by treatment with hydrogen sulphide and precipitation with petroleum from the ether solution. The compound was described as 'extremely hygroscopic' white crystals, $C_3H_4O_4$, 'either hydroxypyruvic acid, tartronic semialdehyde, or dihydroxyacrylic acid'. Fenton & Wilks (1912) favoured the view that this was hydroxypyruvic acid, but their evidence (based on the formation of glyceric acid by treatment with potassium cyanide of the lead salt derived from the crude glyceric acid oxidation mixture) is equivocal.

In order to clear up this confused situation, we have prepared, from the recrystallized lithium salt, analytically pure hydroxypyruvic acid monohydrate (III), which proves to be a white, non-hygroscopic solid of m.p. 82° . The properties of this acid ($C_3H_4O_5$) thus differ markedly from those of the product obtained by Fenton & Wilks. Infrared spectroscopy (see Addendum) indicates that the monohydrate of free hydroxypyruvic acid (unlike the salts) probably exists as the ketonic acid, two carbonyl groups—those of the keto and carboxylic groups—being detectable. The water molecule is, however, quite firmly attached and only slowly removed by heating in high vacuum over phosphoric oxide.

The identity of the lithium salt and free acid as

being hydroxypyruvate was shown: (a) by the quantitative reduction of both compounds (to glyceric acid) by reduced diphosphopyridine nucleotide (DPNH) in the presence of both the lactic dehydrogenase of muscle and the specific hydroxypyruvate dehydrogenase of parsley leaves (Dickens & Williamson, 1958); (b) by quantitative decarboxylation with yeast carboxylase (cf. Dickens & Williamson, 1956b); (c) by means of a colour reaction with naphtharesorcinol (Dickens & Williamson, 1958); (d) by preparation of crystalline derivatives (see below).

The preparation of lithium hydroxypyruvate was mentioned in an earlier brief note (Dickens & Williamson, 1956a). The enzymic behaviour of these compounds will be presented in a forthcoming publication (cf. Dickens & Williamson, 1956b).

The infrared absorption is considered by Bellamy & Williams (see Addendum) to suggest that ionized solutions of both hydroxypyruvate and pyruvate will contain the hydrated derivatives rather than the free carbonyl-containing ketonic acids, whereas the latter may predominate in the un-ionized forms, such as will tend to be present at acidic reactions. This fact may be of importance in the enzymic behaviour of this group of substances. For muscle lactic dehydrogenase, Loewus, Ofner, Fischer, Westheimer & Vennesland (1953) have shown the transfer of hydrogen from one stereoisomer of DPNH to pyruvate to yield the resulting optically active lactate. Accepting a similar type of mechanism for hydroxypyruvate, it may be suggested that two different types of mechanism of reduction may occur, according to whether the hydrated pyruvate ion or the undissociated acid is the

Fig. 2. Possible reaction mechanisms in the reduction of pyruvate and hydroxypyruvate. $\text{R}=\text{CH}_3$ in pyruvate, $\text{CH}_2\cdot\text{OH}$ in hydroxypyruvate; F =lactic dehydrogenase of yeast ('cytochrome b_5 '). References to enzymes: a, Meister (1952); b, Stafford *et al.* (1954); c, Appleby & Morton (1954); Boeri, Cutolo, Luzzati & Tosi (1955); d, Dickens & Williamson (1956a); the highly purified enzyme kindly supplied by Professor E. Boeri also reacts with hydroxypyruvate.

reactant, the latter being presumably that favoured at acid pH values (see Fig. 2): the actual choice of pathways in Fig. 2 is somewhat arbitrary but is based roughly on the respective pH optima of the enzymes concerned.

EXPERIMENTAL

The melting points are uncorrected. All analyses are by Weiler and Strauss, Oxford. Pyruvic acid (British Drug Houses Ltd.) was twice fractionally distilled *in vacuo* and then it solidified readily in the refrigerator. Sodium [3-¹⁴C]pyruvate and sodium [2-¹⁴C]pyruvate were purchased from the Radiochemical Centre, Amersham, Bucks., with specific activity of approx. 2 mc/m-mole. Paper chromatography was on Whatman no. 1 paper with descending-liquid phase.

Preparation of 3-bromopyruvic acid (I). This is described for [3-¹⁴C]bromopyruvic acid: the same method has been used for the [2-¹⁴C]acid and (on four times the scale described below) for the unlabelled acid.

When the method of Sprinson & Chargaff (1946) was first used without modification, a long lag period was followed by a violently explosive bromination, with our materials. To avoid this danger, the addition of a single drop of conc. H₂SO₄ (cf. Ward, 1923) was found entirely satisfactory.

Twice-redistilled pyruvic acid (4.4 g., 50 m-moles) was added to the required quantity (e.g. 100 μC of specific activity 2.34 mc/m-mole) of sodium [3-¹⁴C]pyruvate followed by 0.05 ml. of conc. H₂SO₄. With stirring and protection from moisture, 2.5 ml. of dry Br₂ was added slowly drop by drop while the flask was maintained in a bath at 50°. Under these conditions, the reaction began at once, with decolorization of the Br₂ as added and smooth evolution of HBr. The pale-yellow reaction mixture, after removal of HBr in a desiccator, set to a crystalline mass (97% yield). After one recrystallization from hot dry CHCl₃ (1 ml./g.) colourless crystals, m.p. 58°, were obtained, having 70% of the initial total radioactivity (mother liquors were discarded).

Preparation of lithium hydroxypyruvate monohydrate (II). Bromopyruvic acid (10 g., 60 m-moles) was dissolved in 100 ml. of water and n-LiOH was run in slowly with stirring until a pink colour to phenolphthalein persisted for 10 min. (approx. 90% of that calc. for 2 moles of LiOH/mole of bromopyruvic acid). When this stage of neutralization was reached, 2 g. of activated charcoal was added, the filtrate adjusted to pH 5 by a few drops of acetic acid and concentrated under reduced pressure in N₂ to approx. 20 ml., when colourless crystals began to appear. These were collected after keeping the concentrate at 0° overnight, washed with 10 ml. of ice-cold water and dried over P₂O₅ *in vacuo* at room temperature: yield 3.7 g. (49%). In spite of the loss on crystallization, attempts to recover pure material from the mother liquors were unsuccessful, no doubt because of the changes occurring on concentration of the aqueous solution (see below). Owing to the serious loss on recrystallization, lithium [14C]hydroxypyruvate was generally used in this form without further purification (80% of specific activity of bromopyruvic acid taken).

For recrystallization, the salt (II, 3.7 g.) was dissolved in 14 ml. of water at 65°, quickly centrifuged, and the decanted solution was kept at 0° overnight. Yield of colourless crystals after drying *in vacuo*, 1.65 g. [Found: C, 28.2;

H, 3.9; Li, 5.36. CH₂(OH)·C(OH)₂·CO₂Li requires C, 28.1; H, 3.9; Li, 5.4%. Loss of wt. in high vacuum over P₂O₅: at 60°, no loss; at 110°, 0.0, 2.6 (two specimens). Calc. for 1 H₂O, 14.0%; for 1 CO₂, 34.3%].

The infrared absorption of this substance is described in the Addendum, and the colorimetric and enzymic assay of its content of hydroxypyruvic acid in the following paper (Dickens & Williamson, 1958). Its biological behaviour (cf. Dickens & Williamson, 1956b) will be fully reported later.

Preparation of hydroxypyruvic acid monohydrate (III). A solution of analytically pure lithium hydroxypyruvate monohydrate (500 mg.) in water (20 ml.) was shaken for 10 min. with Amberlite IR-120 (H⁺ form; 5 g.). The filtrate and 10 ml. of water washings were combined and freeze-dried; the residual colourless syrup was kept over P₂O₅ in an evacuated desiccator. It was then dissolved in 15 ml. of ether at 0°, to the clear solution light petroleum (b.p. 60–80°) was slowly added to give a turbidity, with slight further additions over 2 days at 0°. The oil which first separated had then partially solidified and the supernatant liquid was decanted and discarded. To the solution of the precipitate in 15 ml. of acetone an equal volume of light petroleum (b.p. 60–80°) was added. After several days at 0°, the crystalline precipitate was collected and dried *in vacuo* over H₂SO₄ in the presence of paraffin-wax shavings. Yield, 160 mg. *Hydroxypyruvic acid monohydrate* had m.p. 81–82° (vigorous decomp.) [Found: C, 29.6; H, 4.90. CH₂(OH)·CO·CO₂H₂O requires: C, 29.5; H, 4.92%. (The anhydrous acid, C₃H₄O₄, would require C, 34.6; H, 3.84%). Loss of wt. at 0.05 mm. Hg over P₂O₅ at 56°: 2 hr. 8.0; 4 hr. 9.4; 6 hr. 11.1; 8 hr. 11.2. Calc. for 1 H₂O, 14.75%]. On titration in N₂ with Ba(OH)₂ and phenolphthalein, an equivalent wt. of 111 was obtained (calc. 122); the low value is doubtless due to alkaline decomposition (see below). After drying for 8 hr. as described above, the m.p. (85°, decomp.) showed little change, although decomposition is shown by the loss of weight which had occurred.

The infrared absorption spectrum of this compound is reported in the Addendum.

Preparation of the 2:4-dinitrophenylhydrazone of hydroxypyruvic acid (IV) from the lithium salt. Lithium hydroxypyruvate monohydrate (II; 50 mg.) was dissolved in 2 ml. of water and 20 ml. of a cool saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl was added. The yellow crystals which separated almost immediately were collected after keeping at 0° overnight, washed with water and dried *in vacuo* over P₂O₅. Yield, 105 mg. (92%), m.p. crude 135–138° (decomp.); after recrystallization from ethyl acetate–light petroleum (3:1) the m.p. was 160–161° (decomp.) (Sprinson & Chargaff, 1946, give m.p. 162°). After electrolytic reduction (for which we are indebted to Dr I. Smith) and paper chromatography in butanol–acetic acid–water mixture (4:1:5, by vol.) the presence of serine (V) (cf. Sprinson & Chargaff, 1946) was identified by comparison with a marker.

Attempted preparation of a phenylosazone from lithium hydroxypyruvate. The lithium salt (1 m-mole) was dissolved in n-HCl (1 equiv.) and phenylhydrazine (3 m-moles) was added (pH about 5). The solution was kept at 80°; after 5 min. an orange precipitate formed and this was collected after 20 min. at 80°, washed with water and dried (143 mg., 61%, m.p. 115–117°, decomp.). After precipitation from CHCl₃ by light petroleum (b.p. 60–80°), the m.p. was

unchanged (Found: C, 60.2; H, 5.7; N, 18.6. The bisphenylhydrazone $C_{15}H_{14}O_2N_4$ would require C, 63.8; H, 5.0; N, 19.8. The phenylhydrazide of hydroxypyruvic acid monophenylhydrazone (VI), $C_{15}H_{18}O_2N_4$, requires C, 59.7; H, 5.95; N, 18.5 %).

Other variations of this preparation failed to give the crystalline osazone (IX), m.p. 207°, reported by Fenton & Jones (1900): the m.p. was later raised (Fenton & Ryffel, 1902) to 222–224°. It is noteworthy that Sprinson & Chargaff (1946), from their solutions of alkali hydroxypyruvate, were also unable to obtain this compound on the addition of phenylhydrazine acetate, but obtained only 'brown amorphous products' or at higher temperatures 'deeply coloured products' quite unlike the Fenton & Jones osazone.

Attempted preparation of the semicarbazone from lithium hydroxypyruvate. The aqueous solution of recrystallized lithium hydroxypyruvate was treated at room temperature with excess of semicarbazide hydrochloride, with or without the addition of sodium acetate. After several days no precipitation occurred. After evaporation of the solution to small volume only semicarbazide hydrochloride could be recovered. This behaviour also contrasts strongly with that described by Fenton & Wilks (1912) for their oxidation product from glyceric acid, which gave an insoluble substance, m.p. 221°, which they believed (from the analysis only) to be the semicarbazone salt of the semicarbazone of hydroxypyruvic acid,

Although a similar compound did not arise from our preparation of lithium hydroxypyruvate, no difficulty was experienced in preparing a compound of this m.p. from dihydroxyfumaric acid and semicarbazide hydrochloride as described by Fenton & Wilks (1912). For this purpose, recrystallized dihydroxyfumaric acid dihydrate (VII; 5 m-moles) was dissolved with gentle warming in a solution of semicarbazide hydrochloride (20 m-moles in 60 ml. of water). After 16 hr. at room temperature the copious white precipitate (m.p. crude 210°, decomp.) was recrystallized from hot acetic acid, and dried for 30 min. at 100°, at atmospheric pressure; m.p. 227° (decomp.) (Found: C, 26.27; H, 4.43; N, 35.56. Calc. for $C_6H_8O_4N_6 \cdot H_2O$ (X): C, 25.7; H, 4.30; N, 35.9 %). The compound was dried at 80° in high vacuum over P_2O_5 for 5 hr. (Found: C, 26.8; H, 4.17. Calc. for $C_6H_8O_4N_6 \cdot 0.5 H_2O$: C, 26.6; H, 4.0 %). Fenton & Wilks (1912) state that the compound obtained from dihydroxyfumaric (dihydroxymaleic) acid by the above treatment is identical with that obtained from their oxidation product derived from glyceric acid: this latter substance had m.p. 221° after recrystallization from acetic acid and its analysis gave C, 25.4; H, 4.68; N, 35.5. Calc. for $C_6H_8O_4N_6$: C, 25.4; H, 5.07; N, 35.6 %. Fenton & Wilks attributed this to the semicarbazide salt of the semicarbazone. On the other hand, our analysis (above) agrees better with the monohydrated formula X, ($C_6H_{10}O_5N_6$) which loses 0.5 H_2O on further drying. This would suggest that the Fenton & Wilks compound may have been the disemicarbazone of mesoxalic semialdehyde (VIII), containing a molecule of water, which, as our analyses show, is very difficult to remove from this compound. In order to obtain further evidence on this point: (a) the compound, m.p. 227° decomp., was refluxed with water containing a little benzoic acid to which excess of benzaldehyde was added, with a

view to removing the molecule of semicarbazide supposedly present as the salt of the carboxyl group. No decomposition occurred and the unchanged substance (m.p. 215–217°, decomp.; after one recrystallization, m.p. 225°, decomp.) was recovered. (b) The disemicarbazone of mesoxalic acid semialdehyde was prepared as described below, m.p. 225–226° (decomp.), and after drying at 80° *in vacuo* over P_2O_5 gave analysis for C, H and N corresponding closely with the formula $C_6H_8O_4N_6 \cdot 0.5 H_2O$, identical with that obtained on the substance, m.p. 227° (decomp.), prepared from dihydroxyfumaric acid.

It is therefore considered probable that the Fenton & Wilks compound, m.p. 221°, was in fact the hydrated disemicarbazone of mesoxalic acid semialdehyde and not that of the semicarbazide salt of hydroxypyruvic acid semicarbazone, as these authors believed it to be. The precipitation of the phenylosazone (m.p. 209°) of tarttronic acid semialdehyde (XI) occurs immediately when phenylhydrazine reacts at room temperature with XI (Fischer, Baer & Nidecker, 1937). If a similar reactivity to semicarbazide is postulated, this substance (XI) could well be the intermediate in reactions VII → IX and VII → X as is suggested in Fig. 1.

These considerations shed doubt on the purity of the product obtained by oxidation of glyceric acid by Fenton & Wilks (1912), and used by them in preparing the osazone, which neither we nor Sprinson & Chargaff (1946) could obtain from solutions of hydroxypyruvate. Nor does our purified lithium hydroxypyruvate give the insoluble semicarbazide derivative of m.p. 221° obtained by Fenton & Wilks from their material.

Preparation of mesoxalic acid semialdehyde and its derivatives. Dihydroxyfumaric acid dihydrate (VII) was oxidized with $HgCl_2$ at 60° as described by Fenton (1905). After removal of $HgCl$ by filtration and of a trace of excess of Hg^{2+} ions by H_2S , the filtrate and washings were concentrated in an evacuated desiccator over P_2O_5 and NaOH to give a solution of mesoxalic acid semialdehyde (VIII) containing approx. 0.4 m-mole/ml.

Bisphenylhydrazone of mesoxalic acid semialdehyde (IX). This was prepared from a portion of the above solution at room temperature by addition of phenylhydrazine (4 mol. prop.; pH of mixture approx. 5). Immediate precipitation occurred of the yellow osazone in good yield, m.p. crude 147° (decomp.), after recrystallization from hot $CHCl_3$ and a little acetic acid, m.p. 218–220° (decomp.) (Found: N, 20.2. Calc. for $C_{15}H_{14}O_2N_4$: 19.9 %). The m.p.'s recorded from the literature by Fenton & Ryffel (1902) for this much studied osazone ('the osazone of hydroxypyruvic acid') vary from 201–203° to 222–224°. The same compound is obtained by heating dihydroxymaleic acid with phenylhydrazine (Fenton & Ryffel, 1902): by this method we found m.p. 218–219° (decomp.). The product obtained from mesoxalic acid semialdehyde was converted into the pyrazolone (Dakin, 1917) by heating with ethanolic HCl, m.p. 146° (Dakin gives m.p. 150°) (Found: N, 21.0. Calc. for $C_{15}H_{12}ON_4$: N, 21.2 %).

Disemicarbazone of mesoxalic acid semialdehyde (X). This was prepared from a further portion of the same solution of mesoxalic acid semialdehyde. On addition of semicarbazide hydrochloride precipitation occurred immediately (yield, 65 % of that calc. on original wt. of dihydroxyfumaric acid taken); the product, after recrystallization from hot acetic acid, had m.p. 225–226° (decomp.) [Found:

(dried at 80° *in vacuo* over P_2O_5) C; 26.5; H, 4.37; N, 37.2. Calc. for $C_5H_8O_4N_2 \cdot 0.5 H_2O$: C, 26.6; H, 4.0; N, 37.3%. Loss of wt. after 2 hr. at 110° at 0.01 mm. Hg over P_2O_5 : 10.8. 1 H_2O requires 7.7%. After the 2 hr. drying, m.p. was 220° (decomp.); evidently some decomposition had occurred.

Preparation of lithium pyruvate monohydrate. For spectrographic purposes (see Addendum) a sample of lithium salt was prepared from twice redistilled pyruvic acid by a convenient modification of the method of Wendel (1932). The well-stirred aqueous solution of pyruvic acid (0.1 mole in 10 ml.) was titrated with 3.33N-LiOH until just blue to bromothymol blue. Crystals rapidly began to separate, and after 16 hr. at 0° they were collected, washed with a little ice-cold water, and dried *in vacuo* over $CaCl_2$. Yield, 4.2 g. Recrystallization from 15 ml. of water at 90° gave 2.7 g. (Found: C, 32.0; H, 4.4; Li, 5.97. Calc. for $CH_3 \cdot CO \cdot CO_2Li \cdot H_2O$: C, 32.15; H, 4.46; Li, 6.25%. No loss of wt. at 110° in high vacuum over P_2O_5).

Paper chromatography of hydroxypyruvate. Milhaud, Benson & Calvin (1956) have reported two-dimensional paper chromatograms obtained with unpurified $[2-^{14}C]$ -hydroxypyruvate obtained from the bromopyruvate. In phenol-water (4:1, v/v) two distinct spots were obtained, R_F 0.15 and 0.31.

Recrystallized lithium hydroxypyruvate was chromatographed in the above liquid and also in butanol-acetic acid-water (4:1:5, by vol.). The aniline-phosphoric acid mixture of Bryson & Mitchell (1951) used for detection was sprayed on the dried papers, which were then heated for 5 min. at 100°. Single compact purplish-brown spots resulted, obtainable with amounts down to 10 μ g. of hydroxypyruvate, and having R_F in phenol-water 0.08, in butanol-acetic acid-water, 0.22.

Further characterization is possible by means of the dinitrophenylhydrazone, m.p. 160–161°, described above. A solution in 0.01M-phosphate, pH 7.4, was chromatographed with butanol-ethanol-water (Cavallini & Frontali, 1954) as solvent. The hydrazone gave a single yellow spot, R_F 0.32.

Effect of heat on solutions of hydroxypyruvate

Fenton & Wilks (1912) stated briefly that solutions of hydroxypyruvic acid are destroyed by heating on a water bath, a fact which 'sharply distinguishes it from Will's acid' (cf. Will, 1891).

Solutions of lithium hydroxypyruvate were heated at various temperatures and pH values. When the pure aqueous solution of the salt was heated in a boiling-water bath, the reaction rapidly became strongly alkaline owing to lithium carbonate formation, and after 30 min. at 100° only a trace of the original hydroxypyruvate could be detected colorimetrically or enzymically. Paper chromatography of the heated solution showed the presence of a new reducing spot, which was identified as erythrulose (XII) by paper chromatography of the heated deionized solution in phenol-water and in butanol-acetic acid-water (R_F 0.71 and 0.35 respectively; L-erythrulose 0.71 and 0.35; D-erythrulose 0.64 and 0.36). For further characterization the deionized heated solution was evaporated *in vacuo* to a syrup (100 mg. from 640 mg. of lithium salt taken) which was dissolved in 3 ml. of ethanol, filtered and warmed for 5 min. in a boiling-water bath with *o*-nitrophenylhydrazine (110 mg.). The filtered solution was diluted with water, the hot solution

was treated with charcoal, and, on cooling, orange-yellow crystals separated, which were washed with ether, dried and recrystallized from hot water. Yield, 17 mg., m.p. 123–125° (Found: N, 16.1. Calc. for erythrulose *o*-nitrophenylhydrazone 16.5%). The substance appears to be this derivative of DL-erythrulose; the corresponding derivative of L-erythrulose has m.p. 152° (Müller, Montigel & Reichstein, 1937). A portion was decomposed by refluxing with water containing benzoic acid and benzaldehyde. After removal of these substances the solution of the free sugar was chromatographed and run together with authentic L-erythrulose. However, on this particular chromatogram, erythrulose could not be clearly distinguished from erythrulose, so that some erythrulose may possibly have been present. The amount of material was insufficient for a fuller examination. Table 1 (Tube A) shows the estimations of rate of formation of products in the above experiment.

In acid solutions (0.5M-acetate buffer, pH 5.5; 0.1M-acetic acid solution) at 100° decarboxylation also occurs. In the pH 5.5 buffer the solution again, but more slowly, became alkaline, and the rate of breakdown was similar to that of the heated lithium salt solution. In acetic acid it was still slower. Analysis of the heated mixtures (Table 1) was by (a) the colorimetric method (Dickens & Williamson, 1958); (b) by measurement of the decarboxylation with a preparation of yeast carboxylase (Dickens & Williamson, 1956b); (c) by the hydroxypyruvate reductase of Stafford *et al.* (1954). Method (a) estimates both hydroxypyruvate and glycolaldehyde, methods (b) and (c) only hydroxypyruvate. It will be seen from Table 1 that whilst the three methods give closely similar values for the heated aqueous solution of lithium hydroxypyruvate (pH 6.5 → 9), there is a serious discrepancy in the values as obtained colorimetrically after heating at pH 5.5 → 8.1, and still more so in the acetic acid solution, when these are compared with the enzymic assays. This difference is to be attributed to the accumulation of glycolaldehyde (XIII), which does not occur in the series at pH 6.5 → 9.0. That this is the source of the differences found is shown by the figures in parentheses under 'colorimetric' in Table 1. These values are corrected for the presence of glycolaldehyde found after absorption of hydroxypyruvic acid on to Bio-Deminrolit G resin according to the method of Dickens & Williamson (1958). The corrected values for hydroxypyruvate agree quite closely with those determined enzymically.

From these experiments it is clear that, of the reactions

the former decarboxylation proceeds slowly at an acid pH whilst the latter condensation predominates at neutral and alkaline reactions and is then quite rapid. The resemblance of this chemical decarboxylative ketol condensation to the similar enzymic formation of erythrulose from hydroxypyruvate (Dickens & Williamson, 1956b) is quite striking; the main difference is that the enzymic reaction, involving carboxylase and transketolase, yields the pure L-erythrulose, whereas this reaction, of course, gives the DL-form.

Table 1. *Effect of heating aqueous solutions of hydroxypyruvate*

Each of three stoppered test tubes (*A*, *B* and *C*) contained 64 mg. (500 μ moles) of lithium hydroxypyruvate: *A*, in 5 ml. of water; *B*, in 5 ml. of 0.5M-acetate, pH 5.5; *C*, in 5 ml. of 0.1N-acetic acid. The tubes were heated in a boiling-water bath and samples removed for analysis as detailed in the text; colorimetrically and by hydroxypyruvate reductase (see Dickens & Williamson, 1958); by yeast carboxylase and measurement of CO₂ manometrically (see Dickens & Williamson, 1956b).

Results are expressed in μ moles of hydroxypyruvate/ml. of solution. The values in parenthesis are the amounts of hydroxypyruvate found colorimetrically after deduction of the glyceraldehyde present (non-absorbed on Bio-Deminrolit G, see Dickens & Williamson, 1958).

Tube and pH	Time at 100° (min.)	Hydroxypyruvate found (μ moles)		
		Colorimetric method	Carboxylase method	Reductase method
<i>A</i> (pH 6.5 $\rightarrow$ 9)	0	102	93	95
	5	100	90	95
	10	76	74	72
	20	17.5	16.5	16.5
	30	1	3.5	2.5
<i>B</i> (pH 5.5 $\rightarrow$ 8.1)	0	98	95	95
	10	72	48	45
	20	41	16	16.5
	40	17	3	2.5
	60	9	0	0
<i>C</i> (0.1N-acetic acid)	0	98 (95)	93	94
	10	96 (93)	90	86
	20	93 (84)	83	84
	40	86 (71)	73	72
	60	82 (62)	60	60

Action of cold dilute alkali on hydroxypyruvate

Sprinson & Chargaff (1946) reported that solutions of NaOH (0.1–1.5N) acting on hydroxypyruvate yielded an unsaturated compound which, as shown by iodine titration, could be accounted for by a 35–64% formation of the enediol:

We have attempted to prepare this compound (dihydroxyacrylic acid) but without success. When a lithium hydroxypyruvate solution in N-LiOH was kept under N₂ for 1 hr. at 20°, deionized with Amberlite 1R-120(H⁺) and freeze-dried, a solid semicrystalline residue was obtained. This was washed with cold acetone and dried *in vacuo* (yield 30% of wt. of lithium salt taken). It consisted of a non-hygroscopic white solid, m.p. (unsharp) about 85° (decomp.) (Found: C, 30.7; H, 4.2. C₅H₆O₅ requires C, 30.6; H, 4.1%). Titration gave two different values according to the conditions of titration, corresponding to equiv. wts. of 226 and 522 respectively; the lower value was obtained with Ba(OH)₂ in N₂ and the higher with alkali hydroxide in air. The substance is thus not dihydroxyacrylic acid, and in agreement with this did not decolorize iodine solution. The analysis would correspond with that of an addition product of hydroxypyruvic acid and glyoxylic acid (mol.wt. 196), or a higher polymer of similar composition. It was not further investigated.

SUMMARY

1. The preparation of crystalline lithium hydroxypyruvate is described, together with the application of the method for preparing lithium [¹⁴C]hydroxypyruvate.

2. The lithium salt has the analysis of the monohydrate, CH₂(OH)·C(OH)₂·CO₂Li, in which the molecule of water is very firmly bound.

3. Hydroxypyruvic acid, m.p. 81–82° (decomp.), has been prepared from the lithium salt and is also a monohydrate, CH₂(OH)·CO·CO₂H·H₂O; it thus differs from the compound described as hydroxypyruvic acid by Fenton & Wilks (1912).

4. Lithium hydroxypyruvate yields the characteristic dinitrophenylhydrazone, giving serine on reduction. It gives no osazone under the usual conditions, but a crystalline derivative, which may be the phenylhydrazone of the phenylhydrazone, is obtainable. The reasons for the apparently different behaviour of the Fenton & Wilks compound are discussed. It is suggested that the semicarbazide compound obtained by these authors may have been the hydrated disemicarbazone of mesoxalic acid semialdehyde and not, as they assumed, the semicarbazide salt of hydroxypyruvic acid monosemicarbazone.

5. The nature of lithium hydroxypyruvate and hydroxypyruvic acid have been investigated colorimetrically, enzymically, chromatographically and (see Addendum) by infrared spectrophotometry.

6. When aqueous solutions of hydroxypyruvate are heated, carbonate is liberated and a reducing substance which is believed to be DL-erythrulose has been demonstrated as a major product. The rate of decomposition of hydroxypyruvate at 100° and various pH ranges has been studied. In acidic

solutions the thermal decomposition is slower and the main product then reacts as glycolaldehyde.

7. The action of N-alkali on hydroxypyruvate yields an unidentified polymerized decomposition product $(C_5H_8O_8)_n$ which is not the unknown dihydroxyacrylic acid.

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REFERENCES

- Appleby, C. A. & Morton, R. K. (1954). *Nature, Lond.*, **173**, 749.
- Ballou, C. E. & Hesse, R. (1956). *J. Amer. chem. Soc.* **78**, 3718.
- Boeri, E., Cutolo, E., Luzzati, M. & Tosi, L. (1955). *Arch. Biochem. Biophys.* **56**, 487.
- Bryson, J. L. & Mitchell, T. J. (1951). *Nature, Lond.*, **167**, 864.
- Cavallini, D. & Frontali, N. (1954). *Biochim. biophys. Acta*, **13**, 439.
- Dakin, H. D. (1917). *Biochem. J.* **11**, 79.
- Dickens, F. & Williamson, D. H. (1956a). *Nature, Lond.*, **178**, 1118.
- Dickens, F. & Williamson, D. H. (1956b). *Nature, Lond.*, **178**, 1349.
- Dickens, F. & Williamson, D. H. (1958). *Biochem. J.* **68**, 84.
- Fenton, H. J. H. (1905). *J. chem. Soc.* **87**, 813.
- Fenton, H. J. H. & Jones, H. O. (1900). *J. chem. Soc.* **77**, 69.
- Fenton, H. J. H. & Ryffel, J. H. (1902). *J. chem. Soc.* **81**, 426.
- Fenton, H. J. H. & Wilks, W. A. R. (1912). *J. chem. Soc.* **101**, 1570.
- Fischer, H. O. L., Baer, E. & Nidecker, H. (1937). *Helv. chim. acta*, **20**, 1226.
- Haba, G. de la, Leder, I. G. & Racker, E. (1955). *J. biol. Chem.* **214**, 409.
- Horecker, B. L., Smyrniotis, P. Z. & Klenow, H. (1953). *J. biol. Chem.* **205**, 661.
- Ichihara, A. & Greenberg, D. M. (1957). *J. biol. Chem.* **224**, 331.
- Loewus, F. A., Ofner, P., Fischer, H. F., Westheimer, F. H. & Vennesland, B. (1953). *J. biol. Chem.* **202**, 699.
- Meister, A. (1952). *J. biol. Chem.* **197**, 309.
- Milhaud, G., Benson, A. A. & Calvin, M. (1956). *J. biol. Chem.* **218**, 599.
- Müller, H., Montigel, C. & Reichstein, T. (1937). *Helv. chim. acta*, **20**, 1468.
- Sallach, H. J. (1955). In *Amino Acid Metabolism*, p. 782. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
- Sallach, H. J. (1956). *J. biol. Chem.* **223**, 1101.
- Sprinson, D. B. & Chargaff, E. (1946). *J. biol. Chem.* **164**, 417.
- Stafford, H. A., Magaldi, A. & Vennesland, B. (1954). *J. biol. Chem.* **207**, 621.
- Ward, C. F. (1923). *J. chem. Soc.* **123**, 2207.
- Wendel, W. B. (1932). *J. biol. Chem.* **44**, 717.
- Will, W. (1891). *Ber. dtsch. chem. Ges.* **24**, 400.

ADDENDUM

The Infrared Spectra of Hydroxypyruvic Acid and Related Compounds

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In connexion with the work described in the previous paper we have examined the infrared spectra of pyruvic acid, hydroxypyruvic acid monohydrate and their hydrated lithium salts. The spectra are illustrated in Fig. 1 and are also discussed below.

Pyruvic acid has been previously studied by Randall, Fuson, Fowler & Dangel (1949) and by Thomas (1955), and the spectrum has been reported to be abnormal in that only a single carbonyl frequency is shown despite the presence of the two carbonyl functions. Under reasonably high resolution we find that two peaks are in fact present in the liquid, at 1773 cm^{-1} and 1716 cm^{-1} , although the intensity relation is such that the higher-frequency band appears as a well-defined shoulder on the side of the 1716 cm^{-1} band. In chloroform solution the

two bands are well resolved and occur at 1788 cm^{-1} and 1736 cm^{-1} . The very high frequency of the first of these absorptions is not easy to understand as α -diketones do not normally show appreciable interaction effects (Bellamy, 1954). The most likely explanation would seem to be that the possibility of intramolecular hydrogen bonding between the CO_2H and α -keto groups allows the liquid acid to exist as the monomer in the liquid rather than as the more usual dimer, which would absorb at lower frequencies (Bellamy, 1954; Flett, 1951). This is supported by the behaviour in dioxan solution, in which dimeric structures are usually broken (Flett, 1951). In this case no new high-frequency band is found and the absorption (1769 cm^{-1}) is essentially unchanged. The lower-frequency (keto) band moves to 1733 cm^{-1} and a third band of intermediate

Fig. 1. Infrared absorption of pyruvic and hydroxypyruvic acids and of their lithium salts. The spectrum of pyruvic acid is that of two capillary films of different thickness. The spectra for the remaining three substances are for mulls in Nujol, the region of absorption due to the Nujol being shown by the dotted lines. The solid line beneath each dotted section is the spectrum of the substance as a mull in Fluorube (a fully fluorinated hydrocarbon having no absorption in the dotted regions).

frequency appears at 1753 cm^{-1} . This is typical of the frequency which would be expected from a monomeric acid bonded intermolecularly to the solvent rather than intramolecularly to itself. Further, the intensity changes in this band and that at 1733 cm^{-1} which follow alterations in concentration or in solvent indicate clearly that the intensity of the new band increases when that of the 1770 cm^{-1} band diminishes, and vice versa, whereas the intensity of the 1733 cm^{-1} absorption remains essentially unchanged. Finally, the principal OH stretching frequency of the liquid acid is at 3220 cm^{-1} which is much higher than is usual in dimers (Bratoz, Hadzi & Sheppard, 1956). Pyruvic acid can therefore be regarded as a normal α -keto acid and the unusual features of its infrared spectrum can be attributed to its existence in the liquid state as an intramolecularly bonded monomer.

The spectrum of the hydrated lithium salt of pyruvic acid is in marked contrast with that of the parent acid. No carbonyl absorptions are shown but the characteristic CO_2^- stretching bands of the ionized acid are well marked at 1608 cm^{-1} and 1435 cm^{-1} . In addition to the changes to be expected from the removal of the un-ionized carboxyl group, marked alterations occur in other regions of the spectrum. In particular, strong bands appear from 1100 to 1000 cm^{-1} , a region in which bands arising from OH deformation modes commonly occur. In the high-frequency region, bands due to OH stretching modes are found at 3100 cm^{-1} (shoulder) and at 2700 cm^{-1} with a complex pattern of bands in the range 2700 – 2000 cm^{-1} . These are characteristic of very strongly bonded hydroxyl groups and are not commonly found in ionized acids. Also they occur at very much lower frequencies than is normally associated with water of crystallization (see below). The absence of any ketonic function and the presence of OH groups is consistent with the structure $\text{CH}_3\cdot\text{C}(\text{OH})_2\cdot\text{CO}_2^-\text{Li}^+$ and excludes the alternative formulation $\text{CH}_3\cdot\text{CO}\cdot\text{CO}_2^-\text{Li}^+\cdot\text{H}_2\text{O}$.

The spectra of the hydrates of hydroxypyruvic acid and its lithium salt are essentially parallel to the above. In the solid acid two distinct carbonyl absorptions can be clearly identified at 1757 cm^{-1} and 1730 cm^{-1} , which probably originate in the carboxylic acid group (monomer) and ketonic carbonyl respectively. In the high-frequency region four bands are found above 3000 cm^{-1} . Of these the high-frequency bands at 3509 cm^{-1} and 3448 cm^{-1} probably originate in the CH_2OH group and possibly also in the monomeric CO_2H , whereas the lower-frequency bands at 3333 cm^{-1} and 3205 cm^{-1} probably arise from the asymmetric and symmetric stretching modes of the associated water molecule. The positions of bands from hydrated water in this region vary to some extent with the

strengths of the hydrogen bonds by which the water is attached, but two frequencies in this region are shown, for example, by many hydrated inorganic salts and similar materials (Lecomte, 1953). Finally, a band is present at 1623 cm^{-1} which is absent from the spectrum of pyruvic acid. This is the OH deformation frequency of bonded water. The spectrum of this acid therefore indicates that it retains its ketonic character in the solid state.

In the hydrated lithium salt of hydroxypyruvic acid there are again drastic modifications of the spectrum. In the high-frequency region the absorptions at 3120 cm^{-1} and 2703 cm^{-1} and the series of minor peaks between 2700 and 2000 cm^{-1} reappear as in lithium pyruvate monohydrate. These are almost certainly OH stretching modes and combination bands associated with the $-\text{C}(\text{OH})_2-$ group. In addition, two bands appear at 3436 cm^{-1} and 3311 cm^{-1} . These are absent from the spectrum of lithium pyruvate, and probably both of them are associated with the $\text{CH}_2\cdot\text{OH}$ end-group. The origin of the doubling is not clear but crystal-splitting effects may be involved. As before, no ketonic carbonyl absorptions can be detected and the strong CO_2^- bands are shown at 1605 cm^{-1} and 1429 cm^{-1} . This spectrum is therefore consistent only with the structure

It would therefore seem that a clear-cut distinction exists between the nature of any bound water in the free acids and their lithium salts. In the former any hydration which occurs does so as water of crystallization, whereas in the latter addition occurs across the carbonyl link to form the $-\text{C}(\text{OH})_2-$ group. This is not unexpected in view of the considerable differences in the inductive effects of the ionized and un-ionized carboxyl group. In compounds such as chloroacetone and fluoropyruvic acid, the electronegativity of the CH_2Cl and CH_2F groups is insufficient to lead to hydration across the carbonyl link, and in the latter the ketonic absorption is clearly seen in the infrared spectrum as it is shifted to higher frequencies (L. C. Thomas, personal communication). On the other hand, in compounds such as chloral hydrate and α -thenoyl-trifluoroacetone hydrate which we have also studied, the electronegativities of the CCl_3 and CF_3 groups are sufficient to lead to addition across the $\text{C}=\text{O}$ bond and the total disappearance of the appropriate carbonyl absorption from the infrared spectrum.

It should be noted, however, that the distinction between the free acids and their salts which we have observed is primarily of importance in the solid state. In aqueous solutions some degree of ionization of the carboxyl group will occur which would at once be followed by hydration of the ketonic

grouping, to the diol. Hydroxypyruvic acid in aqueous solution might therefore be expected to have a potential ability to react in either of the two forms

Experimental. Spectra were measured with a Grubb Parsons S. 3A double-beam spectrometer with a sodium chloride prism. Samples were examined as capillary films or as thin mulls in paraffin and in fluorinated paraffin oil.

SUMMARY

1. The infrared-absorption spectra of pyruvic acid, hydroxypyruvic acid monohydrate and their monohydrated lithium salts are reported.

2. Pyruvic acid can be regarded as a normal α -keto acid except for features in its spectrum suggesting intramolecular bonding.

3. Lithium pyruvate monohydrate has a quite different spectrum, no ketonic carbonyl absorption being detectable but CO_2^- stretching bands are well marked, as well as bands characteristic of strongly bonded hydroxyl groups. The spectrum is con-

sistent with the formulation $\text{CH}_3\cdot\text{C}(\text{OH})_2\cdot\text{CO}_2^-\text{Li}^+$ and not with $\text{CH}_3\cdot\text{CO}\cdot\text{CO}_2^-\text{Li}^+\cdot\text{H}_2\text{O}$.

4. The spectra of the hydrates of hydroxypyruvic acid and of its lithium salt are parallel with the above, and indicate that whereas the free acid retains its ketonic character in the solid state and the hydration is present as water of crystallization, the lithium salt shows no ketonic carbonyl absorption and its spectrum is consistent only with the structure $\text{CH}_2(\text{OH})\cdot\text{C}(\text{OH})_2\cdot\text{CO}_2^-\text{Li}^+$.

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REFERENCES

- Bellamy, L. J. (1954). *The Infrared Spectra of Complex Molecules*. London: Methuen.
 Bratoz, S., Hadzi, D. & Sheppard, N. (1956). *Spectrochim. Acta*, **8**, 249.
 Flett, M. St. C. (1951). *J. chem. Soc.* p. 962.
 Lecomte, J. (1953). *J. Chim. phys.* **50**, 53.
 Randall, H. M., Fuson, N., Fowler, R. G. & Dangel, J. R. (1949). *The Infrared Determination of Organic Structures*. New York: Van Nostrand.
 Thomas, L. C. (1955). *Nature, Lond.*, **175**, 424.

The Determination of Hydroxypyruvate and Glycolaldehyde

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In order to investigate the biological occurrence and function of hydroxypyruvate, suitably sensitive methods for its determination are essential. Hitherto the only qualitative colorimetric reaction described for this substance has been the purple colour with alkaline ferric salts (Fenton & Jones, 1900), but this proved unsuitable for quantitative purposes and similar colours are given by other hydroxyl compounds. The lack of a stable solid preparation of the acid or its salts, for use as an analytical standard, has been overcome by the preparation of the monohydrates of lithium hydroxypyruvate and hydroxypyruvic acid (Dickens & Williamson, 1958). The analytically pure recrystallized lithium salt, $\text{CH}_2(\text{OH})\cdot\text{C}(\text{OH})_2\cdot\text{CO}_2\text{Li}$, provides the most suitable standard. By the use of this material a satisfactory colorimetric assay has been developed, based upon the grass-green colour produced on heating hydroxypyruvate solutions with naphtharesorcinol in 23N-sulphuric acid (Dickens & Williamson, 1956a). Glycolaldehyde

gives the same colour, but may be distinguished by its non-absorption on a mixed-bed resin, as is described below.

A specific enzymic reaction of hydroxypyruvate, which like the above reaction clearly distinguishes it from pyruvate, has been described by Stafford, Magaldi & Vennesland (1954). In the presence of a hydroxypyruvate reductase extracted from parsley leaves, reduction of hydroxypyruvate by reduced diphosphopyridine nucleotide (DPNH) can be followed photometrically. This stoichiometric reaction has been used to check the purity of our preparations of hydroxypyruvate (Dickens & Williamson, 1958), and its application to determinations in biological material is described below.

In solutions containing both pyruvate and hydroxypyruvate, the total keto acid present may be determined by the oxidation of DPNH in the presence of L-lactic dehydrogenase of muscle, since this enzyme reacts equally well with both substrates (Meister, 1952).